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SYNAPTIC UNCOUPLING IN THE MOUSE COCHLEA INDUCED BY NOISE OR CISPLATIN AND ITS CLINICAL RELEVANCE

Evangelia Tserga

Karolinska Institutet

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Synaptic uncoupling in the mouse cochlea induced by noise or cisplatin and its clinical relevance

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Evangelia Tserga

Principal Supervisor:
Professor Barbara Canlon
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Experimental Audiology

Co-supervisor(s):
Senior researcher Christopher R. Cederroth
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Experimental Audiology

Opponent:
Professor, Rochellys Diaz Heijtz
Karolinska Institutet
Department of Neuroscience

Examination Board:
Associate Professor, Erik Berninger
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology

Professor, Oliver Söhnlein
Karolinska Institutet
Department of Physiology & Pharmacology

Docent, Zhe Jin
Uppsala University
Department of Neuroscience

To my parents
and my sister

‘‘*Επιστήμη ποιητική ευδαιμονίας’’*

Πλάτων

ABSTRACT

The cochlea has an autonomous circadian clock that controls auditory function and increases vulnerability to noise trauma during the active phase. The mechanisms regulating the circadian control of the auditory system are poorly understood. Furthermore, it remains unclear whether the circadian vulnerability to noise also applies to other auditory insults such as cisplatin, an anticancer drug with strong ototoxic side-effects. Here, we investigated the interplay between glucocorticoids or glutamate homeostasis and noise- or cisplatin-induced hearing loss in the context of circadian influences. Circulating glucocorticoids are important synchronizers of clock rhythms and play a role in noise vulnerability. Our results showed that the removal of adrenal glands, and the subsequent suppression of circadian glucocorticoid rhythms, rescued the increased sensitivity to noise exposure at nighttime. The greater vulnerability to night-noise trauma was associated with a glucocorticoid-dependent regulation of inflammatory genes in the cochlea. Dexamethasone, a synthetic glucocorticoid receptor agonist, protected from noise damage when administered at daytime, coinciding with endogenous circulating glucocorticoid levels being low, but was not equally effective at nighttime, highlighting the relevance of considering the time of the day for assessing the effectiveness of a drug. In parallel, we hypothesized that disruption of the auditory glutamatergic synapse could exacerbate the circadian impact of auditory insults. In a first step, we characterized the synapse status in mice lacking the glutamate transporter GLAST and found that GLAST deficient mice have a pre-existing auditory synaptopathy, which led to an increased sound-evoked activity in the inferior colliculus, something that was not seen in wild-type mice. GLAST deficient mice also showed greater vulnerability to cisplatin-induced ototoxicity at nighttime, with a near complete loss of synaptic coupling to the inner hair cell. This effect could be due to the direct impact of cisplatin on the clock machinery since the administration of cisplatin on *ex-vivo* cochleae at nighttime altered the rhythms of the clock protein Period2 in a dose dependent manner. A meta-analysis identified five genes that are associated with cisplatin-mediated ototoxicity in cancer patients. Four of these were found circadian in the mouse cochlea, also suggesting their involvement in the circadian vulnerability to cisplatin. Overall, considering chronopharmacological approaches to cisplatin treatment could diminish the adverse side-effects on the auditory system and improve the life quality of cancer survivors.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications that are referred in the text by their Roman numerals:

I. Cederroth CR*, Park JS*, Basinou V*, Weger BD, **Tserga E**, Sarlus H, Magnusson AK, Kadri N, Gachon F, Canlon B. Circadian Regulation of Cochlear Sensitivity to Noise by Circulating Glucocorticoids. *Current Biology, 2019,* 29(15):2477-2487

II. **Tserga E**, Damberg P, Canlon B, Cederroth CR. Auditory Synaptopathy in mice lacking the glutamate transporter GLAST and its impact on brain activity. Under revision in Progress in Brain Research

III. **Tserga E**, Paublete RM, Björn E, Canlon B, Cederroth CR. Circadian vulnerability of cisplatin-induced ototoxicity in the mouse cochlea. Manuscript

IV. **Tserga E***, Nandwani T*, Edvall NK, Bulla J, Patel P, Canlon B, Cederroth CR, Baguley DM. The genetic vulnerability to cisplatin ototoxicity: a systematic review. *Scientific Report, 2019,* 9(1):3455

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Fontana JM, **Tserga E**, Sarlus H, Canlon B, Cederroth C. Impact of noise exposure on the circadian clock in the auditory system. *J Acoust Soc Am, 2019*, 146(5):3960

II. Sarlus H, Fontana JM, **Tserga E**, Meltser I, Cederroth CR, Canlon B. Circadian integration of inflammation and glucocorticoid actions: Implications for the cochlea. *Hear Res., 2019*, 377:53-60

* Authors contributed equally to this article

CONTENTS

LIST OF ABBREVIATIONS

ABR	Auditory brainstem response
AC	Auditory Cortex
ADX	Adrenalectomy
AN	Auditory nerve
ARHL	Age related hearing loss
CAT	Nucleus of the central acoustic tract
CDDP	Cis-diammine-dichroroplatinum, cisplatin
CN	Cochlear nucleus
CPu	Caudate putamen
CtBP2	C-terminal binding 2 protein
CTR1	Copper transporter 1
DCN	Dorsal cochlear nucleus
DEX	Dexamethasone
DPOAE	Distortion product of otoacoustic emissions
EP	Endocochlear potential
GC	Glucocorticoid
GLAST	Glutamate aspartate transporter
GluA2 (GluR2)	Ionotropic glutamate receptor, subunit A2
GR	Glucocorticoid receptor
IC	Inferior colliculus
ICP-MS	Induced coupled mass spectrometry
IHC	Inner hair cell
IPC	Inner phalangeal cell
LL	Lateral lemniscus
LSO	Lateral superior olive
MeMRI	Manganese enhanced magnetic resonance imaging
MGB	Medial geniculate body
MVe	Medial vestibular nucleus
MSO	Medial superior olive
NFkB	Nuclear-factor kappa B

NIHL	Noise induced hearing loss
OC	Organ of Corti
OHC	Outer hair cell
qRT-PCR	Quantitative real time PCR
PBS	Phosphate-buffered saline
PER2::LUC	Period2 Luciferase
PFA	Paraformaldehyde
ROS	Reactive oxygen species
SCN	Suprachiasmatic nucleus
SGN	Spiral ganglion neuron
SNHL	Sensorineural hearing loss
SOC	Superior olivary complex
SPO	Superior paraolivary nucleus
SV	Stria vascularis
Tz	Nucleus of trapezoid body
VCN	Ventral cochlear nucleus

1 INTRODUCTION

1.1 THE AUDITORY SYSTEM

1.1.1 The peripheral auditory system

The inner ear is responsible for encoding sound waves into electrical information, which is then conveyed to the central auditory system and leads to sound perception. The cochlea is the auditory component of the inner ear, which contains the sensory organ called organ of Corti (OC). The function of the cochlea is to transform sound into electrical signals that are then transmitted to the brain via the auditory nerve. In the OC, there are two types of sensory cells, the inner hair cells (IHC) and the outer hair cells (OHC). There are three rows of OHCs and one row of IHCs. IHCs are responsible for detecting sounds and convert mechanical energy into nerve impulses and OHCs function as cochlear amplifiers and are controlled by efferent nerve activity (Purves, 2018). Sound waves, which are transmitted by the perilymph (extra-cellular cochlear fluid) cause a displacement in the basilar membrane and subsequently the tectorial membrane on which the OHCs are anchored. This movement causes a deflection of OHC stereocilia (hair-like bundles of the sensory cells), which modulates their electro-motility and amplifies the basilar membrane vibration forcing the stereocilia on the IHCs to become deflected, leading to depolarization of the cell and generation of nerve impulses that are relayed to the afferent neurons (Purves, 2018).

The active zone of afferent synapse in mammals is called the synaptic ribbon, first described by Smith and Sjöstrand, 1961 and it is found in both hair cell types, OHCs and IHCs (Khimich et al., 2005; Smith et al., 1961). The main structural component of ribbons is the protein RIBEYE which is involved in the formation of synaptic ribbon scaffold (Schmitz, 2009). The ribbon synapses of IHCs are glutamatergic and near 200 vesicles are tethered to each ribbon (Moser et al., 2006). Release of glutamate triggers the activation of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, made of ionotropic glutamate receptor GluR2/3 and GluR4 subunits, in the postsynaptic terminals (Matsubara et al., 1996) and creates and action potential that propagates to spiral ganglion neurons (SGN) (Goutman et al., 2015) **(Figure 1).** The SGNs are classified in two types, Type-I and Type-II afferent neurons. Recent single-cell RNA-sequencing analyses revealed three novel subclasses of Type-I neurons, Ia, Ib and Ic (Petitpre et al., 2018; Shrestha et al., 2018).

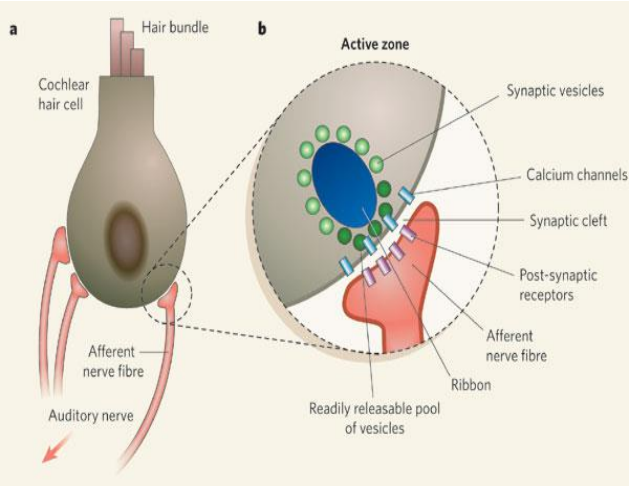

Figure 1: Sound transduction in the peripheral auditory system. a) The hair bundle on the hair cell is activated by sound induced vibrations. The signal is transduced and passed via the afferent nerve fiber and the auditory nerve towards the brain, where it is perceived as sound. **b)** The signal in the hair cell controls calcium channels, causing calcium influx from outside the cell. Consequent changes in the cell's active zone result in neurotransmitter release into the synaptic cleft. Aggregate of neurotransmitter-containing vesicles are bonded to a structure called the synaptic ribbon, localized to concentrated sites of calcium influx (Parsons, 2006).

The stria vascularis (SV) is highly metabolic and provides the cochlea with its blood supply. This vascularized epithelial tissue in the lateral wall of the cochlea, consists of three layers of cells, the marginal cells, the intermediate cells and the basal cells. The main role of SV is to use blood potassium to maintain a high potassium environment with positive endocochlear potential (EP) of +80 mV in the endolymph fluid (Patuzzi, 2011; Tasaki et al., 1959). Alterations in the EP and the composition of the endolymph can alter the mechanotransduction efficacy and thus hearing (Fettiplace, 2017).

1.1.2 The central auditory system

Once the sound waves are transformed to neural signals, they travel through the cranial nerve VIII to the structures of the central auditory system where the sound information is further processed. The central auditory system is responsible for the tonotopic mapping and the decomposition of multi-frequency signals from the cochlea into a spatially organized output arranged according to frequencies (Eggermont et al., 2004). Several nuclei, which participate in the transmission of the auditory information, are located between the auditory nerve (AN) and the auditory cortex (AC): the cochlear nucleus (CN), the superior olivary complex (SOC), the lateral lemniscus (LL), the inferior colliculus (IC) and the medial geniculate body (MGB) (Purves, 2018). All the information is propagated to these nuclei through synaptic transmission. Branching of the nerve fibers, which connect the different nuclei, plays an important role in the parallel processing of the auditory system (Peterson et al., 2020).

Descending pathways of the central auditory system travel in parallel with the ascending pathways. These pathways seem to be reciprocal to the ascending, extending from the AC to the hair cells. A significant component of the ascending and descending auditory pathways,

which transfers the general feedback from the AN is the SOC. The SOC is divided into three main nuclei: medial superior olive (MSO), lateral superior olive (LSO) and the medial nucleus of trapezoid body (Tz). The main function of the SOC is binaural processing and sound localization. The MSO detects interaural time differences and the LSO interaural level differences in sound levels between the two ears. There are also strong feedback connections between primary AC and dorsal cochlear nucleus (DCN), as well as from auditory cortical areas via the central nucleus of IC (Coomes et al., 2004). Changes in the cortical activity as a result of a damage or inhibition, can lead to direct changes in the subcortical activity of IC and DCN and even to indirect changes in the cochlea. Subsequently, DCN can affect the processing of lemniscus activity in ventral cochlear nucleus (VCN). Thus, there is a synergy of changes happening between the cortex and the brainstem. Hearing injuries or application of ototoxic drugs create an imbalance between the excitation and inhibition of the neurons in the cortex leading to the reorganization of the tonotopic map, called homeostatic plasticity (Eggermont et al., 2004).

1.2 HEARING LOSS

According to the World Health Organization, over 5% of world's population is suffering from disabling hearing loss and this impairment highly affects the quality of life (WHO). Hearing impairment is now the 4th leading cause in years lived with disability (Wilson et al., 2017). Hearing loss has been shown in many populations to have negative consequences for cognition and mental health, as well as is an important risk factor for dementia (Greenzang, 2018; Thomson et al., 2017). Noise-induced hearing loss (NIHL) can be divided into temporary and permanent and each one of them has different cochlear pathologies. Thresholds can be recovered after temporary hearing loss, while permanent hearing loss is usually resulting in hair cell and neuronal loss and can lead to severe hearing impairment (Ishii et al., 1998). Correspondingly, with increased life expectancy, the prevalence of age-related hearing loss (ARHL) is also on the rise (Roth et al., 2011). Both NIHL and ARHL are known as sensorineural hearing loss (SNHL) because the dysfunction is initiated in the inner ear's cochlea. For decades, it has been known that hair cell damage is a key contributor in SNHL. Emerging evidence also points towards auditory neuron and synaptic damage as a major cause for hearing impairment. In addition to NIHL and ARHL, numerous other etiologies of acquired hearing loss exist, such as heredity, use of ototoxic medication, bacterial or viral ear infections and head injuries (Sha et al., 2017).

1.2.1 Noise overexposure

High-level noise affects the hair cells, reduces cochlear blood supply, damages peripheral synapses (Henry et al., 1995) and SGNs (Liu et al., 2012), as well as, affects the integrity of fibrocytes in the spiral limbus and the spiral ligament, leading in acute and chronic rearrangement of central auditory structures (Hirose et al., 2003; Wang et al., 2002). Hair cells are more sensitive to noise trauma than the supporting cells. NIHL is known to affect the OHC body as well as the stereocilia. The consequence of noise trauma can be OHC loss which can be monitored by measuring otoacoustic emissions. The death of OHC from a noise exposure is more common than IHC loss (Thorne et al., 1985). This occurs because lower amounts of noise are capable of inducing OHC death than what is needed for IHC damage. IHC loss also starts to appear when permanent hearing threshold shifts exist (Liberman et al., 1978; Lim, 1976). Although OHCs are more prone to death after noise exposure compared to IHCs, their stereocilia are less susceptible to noise than the IHC ones (Kaltenbach et al., 1992).

Furthermore, excitotoxicity that damages glutamate receptors at the afferent synapse leads to hearing impairment (Puel et al., 1998). This excitotoxicity likely results from excessive presynaptic release of glutamate at the IHC. As a result, the synaptic ribbons are reduced in the IHCs after noise exposure (Kujawa et al., 2009). On the other hand, it is evidenced that noise exposure causes excitotoxic neural swelling through changes in the mitochondrial activity and free radical production and induces both apoptotic and necrotic cell death in the OC (Henderson et al., 2006; Le Prell et al., 2007). Reactive oxygen species (ROS) formation has been found in marginal cells of SV and in the OHCs (Henderson et al., 2006). Moreover, upregulation of endogenous or exogenous antioxidant factors can cause NIHL attenuation (Le Prell et al., 2007). In addition, an upregulation of pro-inflammatory cytokines and macrophages during the acute phase of inflammation has been found after a noise exposure (Hirose et al., 2005). The sites of inflammatory actions are found primarily in the SV, spiral ligament as well as, in the SGNs (Hirose et al., 2005; Wang et al., 2002).

In general, NIHL shows similarities among different animal models, including humans. For instance, high sensitivity of OHCs appears to be consistent both in animals and in humans (Lim, 1976). Differences do however exist in the amount of exposure, the duration, frequency and the intensity needed to cause hearing loss. Many rodent models are more sensitive to noise exposure compared to humans (Dobie et al., 2017). It is also anticipated that signaling pathways underlying NIHL will be preserved among the different species, allowing for therapeutic strategies to be successful also in humans (Kujawa et al., 2019).

1.2.2 Cisplatin ototoxicity

Cisplatin is a widely used chemotherapeutic agent, mostly effective for solid tumors such as lung, head and neck, testicular and ovarian cancer (Sheth et al., 2017). The main reported side effects of cisplatin treatment are nephrotoxicity, peripheral neurotoxicity and ototoxicity (Callejo et al., 2015). High doses of cisplatin usually affect first the OHCs in the basal part of the cochlea (van Ruijven et al., 2005), inducing hearing loss mainly in high frequencies. Damage in the upper turn, low frequency region of the cochlea can also happen after high dose cisplatin treatment (Lanvers-Kaminsky et al., 2017). This impairs hearing function and can even cause deafness. Cisplatin also damages the SV, the SGNs and the supporting cells (Breglio et al., 2017). The precise mechanism which is responsible for the auditory impairment in these tissues, is not fully understood, but it is assumed that the increased formation of ROS in mitochondria followed by collapse of the oxidative metabolism and apoptosis or necrosis, plays a significant role in cisplatin ototoxicity (Schacht et al., 2012). Interestingly, it has been recently shown that platinum compounds are retained in the SV for a long period (Breglio et al., 2017). Cisplatin ototoxicity has been extensively studied in different animal models. It has been shown that the mammalian high affinity copper transporter 1 (CTR1), encoded by *SLC31A1* gene is a membrane protein that plays an essential role in cisplatin uptake (Holzer et al., 2004). On the other hand, organic cation transporter 2 (OCT2) has been also identified in the hair cells of OC and the cells of SV in mouse cochlea (Ciarimboli et al., 2005) and has been found in pediatric cancer patients to be related with otoprotection (Lanvers-Kaminsky et al., 2015). Furthermore, there are other mechanisms involved in cisplatin induced ototoxicity and which have been presented in experimental animals. P53 and caspase-dependent apoptosis pathway shown to be activated in rats when they are exposed to high cisplatin doses (So et al., 2007; Wang et al., 2004). Additionally, in a recent study, genetic deletion or pharmacological inhibition of p53 in adult mice prevents from cisplatin ototoxicity and improves anti-tumor efficacy (Benkafadar et al., 2017). In the spiral ligament and in SV of the mouse cochlea, increase of nuclear-factor kappa B (NFkB) and induction of nitric oxide synthase (NOS) have been demonstrated after systemic cisplatin administration (Watanabe et al., 2002). Additionally, studies *in vitro*, in HEI-OC1 cells and *in vivo*, in mice have identified the involvement of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in cisplatin mediated ototoxicity (So et al., 2007).

In clinical research, the prevalence of cisplatin ototoxicity is more common in pediatric cancer patients (Yancey et al., 2012). The incidence of cisplatin induced hearing loss is ranged depending on dose, duration, combination of other ototoxic drugs or irradiation, age and gender of the patients, nutritional status, pre-existing hearing impairment and time of treatment (Sheth

et al., 2017). In addition, genetic predisposition factors related to cisplatin ototoxicity have been previously described such as, polymorphisms of antioxidant enzymes (Oldenburg et al., 2007), mitochondrial mutations (Peters et al., 2003) and genes involved in DNA repair (Caronia et al., 2009). However, some polymorphisms were replicated and others not; a comprehensive view of the genetic variants increasing the risk of cisplatin ototoxicity is missing.

1.3 COCHLEAR SYNAPTOPATHY

In SNHL, cochlear hair cells are among the most vulnerable elements, where in most of the cases, degeneration of their nerve fibers is observed, accompanied by changes in hearing thresholds (Wang et al., 2002). In animal models, a delayed loss of SGNs is also detectable (Lin et al., 2011). However, recent work in animal models has revealed that hair cell loss and upregulated threshold shifts are not always the case of a hearing damage but a hidden hearing loss called cochlear synaptopathy can be present in terms of IHC synaptic impairment, damaged synaptic transmission to SGNs and disrupted propagation of sound information towards the auditory nerve (Kujawa et al., 2015). Synaptic damage associated with a reduction of Wave I amplitude of the auditory brainstem response (ABR) has been observed in humans exposed to noise (Bramhall et al., 2017). Wave 1 amplitude changes have also been identified in animal models of noise, aging and gentamycin (Kujawa et al., 2006; Liberman et al., 2015; Ruan et al., 2014). To date, the most common measure of synaptopathy is ABR wave 1 amplitude, although there have been many debates about its reliability, sensitivity and specificity and if there is indeed a direct connection to synaptopathy (Hickox et al., 2017). Several methods to assess synaptopathy are under constant development, such as ABR latency (Mehraei et al., 2016), the envelope following response (EFR) (Shaheen et al., 2015) and the middle ear muscle reflex (Valero et al., 2016).

1.3.1 Glutamatergic neurotransmission and excitotoxicity

A proper glutamatergic neurotransmission is acquired by the fast removal of glutamate, the main neurotransmitter of central nervous system, from the synaptic cleft (Niciu et al., 2012; Rauen et al., 1998). Glutamate excitotoxicity is defined as cell death resulting from the toxic actions, such as excessive stimulation or not adequate clearance of glutamate. The excessive efflux of glutamate causes an overactivation of glutamate receptors, which can result in neuronal dysfunction and cell death. There are three major types of ionotropic glutamate receptors and several classes of metabotropic receptors linked to G-proteins, which are involved

in glutamate neurotransmission. The main ionotropic receptors, activated by glutamate, are the N-methyl-D-aspartic acid (NMDA), the AMPA and the kainic acid (KA) receptors (Dong et al., 2009). Similar excitotoxic environment can be presented in the cochlea. AMPA-type glutamate receptors are responsible for neurotransmitters' release in the IHC synapse (Nouvian et al., 2006; Puel, 1995). NMDA receptors are also expressed and localized between the synapses of the IHCs and the dendrites of SGNs (Ruel et al., 2008). Excess of glutamate or failure of glutamate clearance from the synaptic cleft because of dysfunction of the glutamate receptors or transporters, can lead to swelling of synapses and cochlear nerve terminals by creating an excitotoxic environment (Pujol et al., 1993). Glutamate excitotoxicity can be exacerbated by adding glutamate receptor agonists, after noise exposure or aminoglycoside treatment (Kujawa et al., 2009; Ruel et al., 2007). Moreover, a study suggests that IHC glutamate excitotoxicity may result from impulse noise trauma (Cho et al., 2013). Generally, glutamate excitotoxicity blocks the neural activity and leads to neural degeneration which yields synaptopathy or hearing loss (Moser et al., 2013).

1.3.2 Glutamate aspartate transporter (GLAST)

Glutamate plays an important role in the peripheral auditory system and the central nervous system as excitatory neurotransmitter, but its abundance can lead to unnecessary excitotoxicity, which activates the proper glutamate transporters. Glutamate aspartate transporter (GLAST, also known as EAAT1 or SLC1A3), a membrane glycoprotein, which removes excess glutamate from the extracellular space (Chen et al., 2017), is expressed in cerebellar astrocytes (Takatsuru et al., 2007) specifically in Bergmann glial cells (Schmitt et al., 1997), in cerebral neocortex (Kim et al., 2011) and subsequently in the circumventricular organs (Berger et al., 2000), the retina (Pow et al., 1999) and the inner ear (Furness et al., 1997). GLAST provides maintenance of extracellular environment and stabilizes cell-to-cell communication (Jin et al., 2003). In the cochlea, where it is present, there have been also found 2 more subtypes of Na^+-dependent glutamate transporters: GLT-1 (EAAT2) and EAAC1 (EAAT3) (Chen et al., 2010). GLT-1 and EAAC1 have been identified in SGNs (Furness et al., 2003). GLAST is present in supporting inner phalangeal cells (IPC) surrounding the IHCs and the afferent neuron synapses, in Schwann and satellite cells surrounding the SGNs and in fibrocytes of spiral ligament (Furness et al., 2009). It buffers the excess glutamate release at the synapse (**Figure 2**).

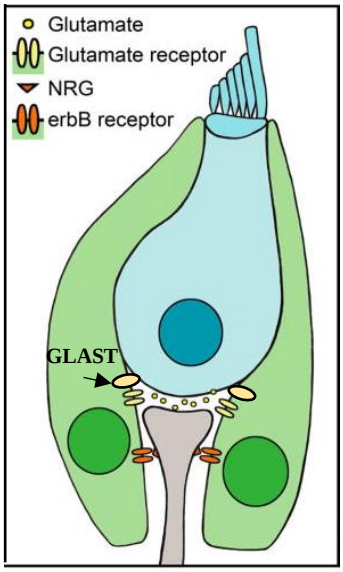

Figure 2: Clearance of glutamate from synapse through glutamate receptors and GLAST. IHC synapse (blue) with SGNs (grey) surrounded by supporting cells (green). SGNs express neuregulin which is tethered to erbB-receptors, located to the supporting cells, promoting the SGN survival. Glutamate is released from IHC and cleared by GLAST and glutamate receptors (Monzack et al., 2013).

Mice lacking GLAST (GLAST KO) exposed to moderate noise levels display a massive synaptic swelling due to the incapacity of the supporting cells to pump back the excessive glutamate which is released by IHCs (Glowatzki et al., 2006; Hakuba et al., 2000). Furthermore, GLAST has also been found to be expressed in human cochlear fibrocytes but not in IPCs (Ahmed et al., 2013).

1.4 CIRCADIAN RHYTHMS

The rotation of the earth around its axis and the sun creates a daily (approximately 24 hours) and seasonal periodicity, which is regulated by biological clocks. The biological clock machinery is an endogenous timing system, capable of generating biological rhythms, called circadian (by Latin *circa Diem*, meaning about a day) even in the absence of environmental cues. Molecular clock imposes a temporal organization of key physiological processes such as behavior, metabolic function, locomotor activity, cell-cycle, reproduction, immune function, cardiovascular and hormonal regulation (Lowrey et al., 2004). The suprachiasmatic nucleus (SCN) of the hypothalamus, is the master clock, which acts as a circadian pacemaker, synchronizes and coordinates rhythms at the organ level to regulate physiological function (Reppert et al., 2002). SCN is unique since it is the only clock that is directly reset by the light received via the retinohypothalamic tract (Hannibal et al., 2002). Because of such characteristic, it succeeds to maintain the robustness and synchronicity of central and peripheral clocks (Ko et al., 2006). The importance of the SCN in synchronizing peripheral organs has been tested in the absence of SCN in lesioned animal models, where peripheral tissues become desynchronized with time and there is significant loss of clock gene expression (Akhtar et al., 2002; Terazono et al., 2003).

In the absence of SCN, glucocorticoids (GC), which are steroid hormones (corticosterone in rodents and cortisol in humans) released by the adrenal glands in a circadian manner, become arrhythmic (Radziuk, 2013). Their higher expression is appeared in the onset of the active phase of each organism (daytime in humans, nighttime in rodents). GCs have been identified in the central nervous system and other organs (Androutsellis-Theotokis et al., 2013), but also have been detected in the inner ear of animals and humans, both in the cochlear and the vestibular part (Erichsen et al., 1996). More specifically, glutamate receptors (GR) have been found in the hair cells, supporting cells, spiral ligament and SV, suggesting a potential role in the regulation of sensory and non-sensory tissues (Kil et al., 2013).

In mammals, the generation of circadian rhythms is a cellular process that includes auto-regulatory transcriptional/translational feedback loops (Albrecht, 2002). The central and peripheral clockwork circuitry contains a negative self-regulated feedback loop consisting of four transcriptional repressor-encoding genes *Per1, Per2, Cry1, Cry2,* which are regulated by the core loop positive BMAL1-CLOCK protein complexes, which in turn will be repressed by PER-CRY complexes resulting in a 24 hour cycle (Albrecht et al., 2003). A secondary interlocking loop involves ROR (RORα, RORβ and RORγ), which are activated by BMAL1-CLOCK complex and REVERB (REVERB-α and REVERB-β) which repress or activate *Bmal1* and *Clock* transcription in a circadian manner (Basinou et al., 2017). These core clock elements are necessary for the generation and maintenance of circadian rhythms (Kolbe et al., 2015; Kondratov et al., 2007).

1.4.1 Circadian rhythms in auditory system

The function of the peripheral auditory organ has been shown to be regulated by circadian mechanisms. Noise has been found to behave as Zeitgeber time (time keeper) for humans, since sound stimulation at nighttime can change circadian rhythms of melatonin and core body temperature (Goel, 2005). The adult mouse cochlea was identified to possess a self-sustained circadian clock that alters cochlear sensitivity to noise at different times of the day (Meltser et al., 2014). Specifically, it has been shown that night noise overexposure affected molecular cochlear rhythms more than day noise and this was associated with greater hearing loss (Meltser et al., 2014). In support of this finding, previous studies in rodents have identified circadian patterns of acoustic startle response (Chabot et al., 1992). Furthermore, antibiotic-mediated ototoxicity appears to be circadian influenced, since more damaging outcomes occurring at nighttime (Soulban et al., 1990; Yonovitz et al., 1991). Notably, high doses of cisplatin has been shown to trigger mortality in a circadian manner, being more toxic at nighttime than

daytime and CLOCK has been found to be overexpressed in cisplatin-resistant cells potentially playing an important role in multidrug resistance through glutathione-dependent redox system (Sun et al., 2017). These studies suggest that cisplatin could have greater impact on cochlear function depending on the time of the day it is administered.

The molecular clock has also been identified in the IC (Park et al., 2016), which plays a significant role in noise-induced pathologies (Berger et al., 2014; Salvi et al., 1990). Circadian dysfunctions of metabolism, hormone regulation or immune responses, affect the homeostasis of the auditory system (Basinou et al., 2017). As it has been evidenced in mouse cochlea, IC is also expressing, in a circadian manner core clock gene such as *Per1*, *Per2*, *Bmal1* and *Reverb-α* (Meltser et al., 2014; Park et al., 2016).

Additionally, it has been shown that the reliability of the distortion product of otoacoustic emission (DPOAE) findings in the cochlear function of human volunteers depends on the time-of-the-day the experiments performed (Cacace et al., 1996). As it seems, the circadian clock significantly influences the stimulus frequency, stimulus sound pressure level and the oral temperature in humans (Cacace et al., 1996).

2 AIMS OF THE THESIS

The main aim of this thesis was to understand the mechanisms by which circadian rhythms modulate hearing loss. This was examined by using two different models of auditory insults: noise trauma and cisplatin ototoxicity.

The following objectives were addressed:

- The contribution of glucocorticoids in the vulnerability to day or night noise exposure.
- The role of a glutamate transporter (GLAST) on the integrity of the auditory synapse and the consequences on the peripheral and central auditory system.
- Whether cisplatin-mediated ototoxicity is influenced by circadian biology in glutamate transporter (GLAST) deficient mice.
- A meta-analysis of genetic variants which are associated with cisplatin ototoxicity in humans.

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

All experimental animal procedures were performed with the guidelines and the regulations of Karolinska Institute and approved by the regional ethical committee (Stockolm's Norra Djurförsöksetiska Nämnd, N156/14 and N140/15). GLAST KO mice, generated by a mix CBA x C57Bl/6 background, provided by Dr Tanaka (Otori et al., 1994) were used for noise exposure and cisplatin administration, audiological assessment, magnetic resonance imaging and molecular evaluation. Mice were 2-6 months old. PERIOD2::LUCIFERASE (PER2::LUC) knock-in mice were generated from a C57Bl/6 background (Yoo et al., 2004) and *Bmal1 (fx/fx)* were provided by Dr Bradfield (Bunger et al., 2005). We further crossed *Bmal1* with PER2LUC mice in order to track cochlear oscillations in the presence or absence of a functional BMAL1. PER2::LUC and CBA/Ca/Sca (from Scanbur) mice were used for noise and audiological evaluation, adrenalectomy, protein and gene expression analysis profile. Mice were 1-4 months old. All mice had *ad libitum* access to food (Lactamin R34, Lantmännen) and water, were housed under a 12:12 h light/dark cycle and the temperature was stable at 19-21℃. Experiments in darkness were performed under red light.

3.2 *IN VIVO* AND *EX VIVO* PHARMACOLOGICAL TREATMENTS

Mice were treated with intraperitoneal injection (i.p.) 0.5 mg/kg Dexamethasone 21 phosphate disodium salt (DEX, Sigma Aldrich, D1159) dissolved in phosphate-buffered saline (PBS) prior to noise exposure (Paper I), once per day for 7 consecutive days prior to noise trauma with 0.24 mmol/kg (30 mg/kg, 200 µl/30 gr body weight) $MnCl_2$ diluted in 0.9 % NaCl (saline) or with only 0.9 % NaCl (as a control) (Yu et al., 2005; Grunecker et al., 2010) (Paper II). In parallel, mice were treated with 4 mg/kg/day cisplatin (Sigma Aldrich, 479306) for 4 consecutive days or nights, diluted in 0.9 % saline or one single dose of 4 mg/kg cisplatin administered either at daytime or at nighttime (Paper III). For *ex vivo* experiments the following drugs were administered: 5 µM DEX dissolved in PBS, 25 µM Mifepristone (RU-486, Sigma Aldrich, M8046), 5 µM Spironolactone (Sigma Aldrich, S3378) dissolved in ethanol, 5 µM Forskolin (Sigma Aldrich, F3917) diluted in PBS (Paper I) and 40-1600 nM cisplatin dissolved in PBS (Paper III).

3.3 NOISE EXPOSURE AND AUDITORY MEASUREMENTS

Mice were transferred awake inside a 225x120x100 cm^3 acoustic chamber, where were placed individually. Sham mice were treated alike but in the absence of noise exposure, placed in a neighboring soundproof chamber. They were treated with either narrowband noise (6-12 kHz) at 100 dB for 1h either at daytime or nighttime (Paper I), or with a narrowband noise (6-12 kHz) at 90 dB for 24h, starting at 9 a.m (Paper II). To ensure that no interruptions or fluctuations in noise presentation had occurred, noise exposure level was recorded during the whole exposure period. After exposure, animals were immediately used either for auditory measurements or manganese enhanced-MRI experiments.

In order to assess hearing quality after noise exposure or cisplatin treatment, we performed DPOAE for evaluating the OHC function and ABR for evaluating synapse function between IHC and SGN. Mice were anaesthetized with a mixture of ketamine (ketaminol 50 mg/ml, Intervet, 511485) and xylazine (Rompum 20 mg/ml, Bayer, KP0A43D), 100 and 10 mg/kg, respectively (body weight 200 µl /30 gr) and placed in a custom-made acoustic chamber with sound absorbing material. Body temperature was monitored and kept at 37 °C during the measurements. First, we recorded DPOAEs. The $2f1 - f2$ distortion product was measured with $f2$ = 8, 12, 16, 24 kHz, $f2/f1$ = 1.25, and stimulus levels $L1 = L2 + 10$ dB SPL. DPOAE thresholds were defined as the lowest level of $f1$ required to produce a DPOAE ≥ -5 dB SPL.

In consequence, we measured ABRs. For each frequency, sound intensity was decreased from 90-5 dB SPL in 5 dB steps, until threshold was reached and confirmed with 2 to 3 replicate measurements. Threshold was defined as the lowest sound intensity with visually identifiable and reproducible waves. Wave 1 amplitude was determined as the difference between the first wave peak and its subsequent trough. ABR and DPOAE measurements were performed 5-7 days prior to noise or cisplatin administration (baseline), 24h and 2 weeks after the trauma. Additionally, ABR measurements were performed 2 weeks after operation of adrenalectomy.

3.4 MANGANESE ENHANCED MAGNETIC RESONANCE IMAGING (MeMRI)

Manganese was used as a contrast agent achieving high resolution imaging (70 µm) using the following methodology, appropriate for evaluating brain activity. On the 7^{th} day, corresponding to the last $MnCl_2$ injection, mice were exposed to the sound or silence for 24h. At the end of the exposure, mice were anaesthetized and fixed through cardiac perfusion of 4% cold paraformaldehyde (PFA). After being decapitated, heads were placed in 50 ml syringes filled

with fomblin oil (SOLVAY, DA 410) and were transferred to the KERIC facility of Karolinska Institute for an overnight MRI scanning.

Insight Toolkit (ITK) Snap software v3.8 (www.itksnap.org) was used for the quantification of manganese enhancement following previously reported recommendations (Yushkevich et al., 2006). Seven anatomical structures related to auditory pathway were identified using the mouse brain atlas (Franklin et al., 2008): DCN (- 5.80 mm to - 6.48 mm), Medial Vestibular nucleus (MVe, - 5.68 mm to - 6.64 mm), LSO (-5.02 mm to -5.68 mm), Superior Paraolivary nucleus (SPO, - 5.02 mm to - 5.52 mm), Tz (-4.84 mm to – 5.68 mm), IC (- 4.16 mm to - 5.34 mm) and nucleus of the Central Acoustic Tract (CAT, - 4.60 mm to - 4.96 mm) which were located caudally from the bregma along the rostrocaudal axis. An additional structure, the Caudate Putamen (CPu, 1.94 mm to -2.30 mm) was identified as a reference independent of sound stimulation (Yu et al., 2005).

3.5 SCN ABLATIONS AND ADRENALECTOMY (ADX)

Bilateral lesions of the SCN were performed in a stereotaxic apparatus (World Precision Instruments, Sarasota, FL) under ketamine/xylazine anesthesia (i.p. 80 and 14 mg/kg). Lesions were made using an electric stimulator (S44, Grass Technologies, Warwick, RI) and stimulus isolation unit (SIU5, Grass Technologies). The sham lesioned mice underwent the same operation, but no electric current was passed through the electrode.

To validate the lesions, SCN lesioned mice and sham operated mice were individually placed in the Comprehensive Laboratory Animal Monitoring System cages (CLAMS™, Columbus Instruments, Columbus, OH). After 24 h of acclimatization to the cages, animals were kept in the system for a period of at least 4 consecutive days. Respiratory metabolism was analyzed with oxygen consumption (VO_2) and respiratory exchange rate (RER).

Bilateral removal of the adrenal glands was performed in anaesthetized mice (100 mg/kg ketamine and 8 mg/kg xylazine) with a dorsal midline incision and lateral retroperitoneal incisions. After surgery, skin was sewed and analgesic drug (0.1 mg/kg Temgesic) was given.

3.6 CORTICOSTERONE ELISA

For the assessment of plasma corticosterone levels in ADX or sham mice, blood was collected around the clock (6 time points) in heparin coated tubes and centrifuged. Plasma was isolated

and corticosterone levels were quantified with and ELISA kit according to the manufacturer's instructions (Corticosterone EIA kit, Enzo, ADI-900-097).

3.7 ORGANOTYPIC CULTURES AND PER2::LUC OSCILLATIONS WITH BIOLUMINESCENCE RECORDINGS

Tissues were dissected from PER2::LUC knock-in mice and cultured organotypically on a membrane (Millipore, PICMORG50) as previously described (Meltser et al., 2014). *Ex vivo* organs were kept in culture for a minimum of 6 days. The bioluminescence emission from each cultured sample was measured for a duration of 1 min every 10^{th} minute with Lumicycle, a microplate luminometer equipped with photomultiplier tubes (Actimetrics, Wilmette, IL). Parameters of PER2::LUC rhythmicity (amplitude, phase and period) were assessed. Amplitude and phase were quantified using Origin software 8.1 SR1 (Microcal Software, Northampton, MA, USA) and period was evaluated by using the Lumicycle Analysis program from where we extracted the raw values. After one day in the culture, *ex vivo* tissues either synchronized with forskolin or they did not. Consequently, they were treated with drug or PBS (control) either at the trough (day 3 of culture) or at the peak (day 3.5) of the oscillation. The drug was washed out 3 days later, when fresh culture medium was added. Analysis was done by following the protocols already published before (Cederroth et al., 2019; Park et al., 2017).

3.8 INDUCED-COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

Platinum levels were measured in OC/SGN, SV and blood from GLAST WT and KO, one hour after a single injection of 4 mg/kg cisplatin, administered either at daytime (08.00 am) or at nighttime (08.00 pm). Mice were euthanized by isoflurane inhalation, followed by decapitation. In order to collect plasma samples, blood was obtained from the trunk after decapitation. For the tissues processing, the analysis and quantification of platinum compounds, the exact protocol of Breglio *et al.,* was followed (Breglio et al., 2017).

3.9 IMMUNOHISTOCHEMISTRY

For immunostaining, mice underwent transcardiac perfusion with 4% PFA and cochleae were decalcified in 0.12 M EDTA for 2-3 days. For the demonstration and quantification of presynaptic ribbons, postsynaptic glutamatergic AMPA-receptors (GluR2) and their coupling, cochlear surface preparations were micro-dissected and stained for a) C-terminal binding

protein 2 (mouse (IgG1) anti-CtBP2, 612044 from BD-Biosciences, used at 1:200), b) GluR2 (mouse (IgG2a) anti-GluA2, MAB397 from Millipore, used at 1:1000) and c) rabbit anti-Myosin VIIa, (25-6790 from Proteus Biosciences, used at 1:200) to delineate the hair cell bodies. Primary antibody incubations were performed overnight at 37^{o}C, followed by 2 hours incubation at 37^{o}C with secondary antibodies coupled to Alexa fluor dyes (IgG1 goat anti-mouse AF568 at 1:500, IgG2a goat anti-mouse AF488 at 1:500 and IgG donkey anti-rabbit AF647 at 1:200), correspondingly. Cochlear frequency mapping, for 6, 8, 12, 16, 24, 32, 48 and 64 kHz along cochlear length, was then performed using a custom plug-in to ImageJ from NIH (Measure_Line.class from the Liberman laboratory at the Eaton-Peabody Laboratory). Confocal z-stacks along discrete regions of the basilar membrane, capturing synaptic structures of around 10 inner hair cells, were made with a 63x oil immersion objective (N.A.1.40) on a Zeiss LSM 880 confocal microscope. Image stacks were analysed using Imaris software (x64 9.2.0, Bitplane AG, Zurich, Switzerland) for the number of ribbons or postsynaptic receptors and their pairing.

3.10 QUANTIFICATION OF mRNA TRANSCRIPTS

For the evaluation of mRNA transcript expression patterns at different time points around the clock, we performed Quantitative real-time PCR (qRT-PCR), Nanostring nCounter assays and RNA sequencing analysis. Detailed description of the above methods is provided in the respective papers (paper I and III). In both qRT-PCR and Nanostring analysis, 6 housekeeping genes were used for picking of the four more regular references for normalization, which was carried out with the geNorm algorithm (Vandesompele et al., 2002).

3.11 STATISTICAL ANALYSIS

All data are presented as mean ± SEM. One or two-way ANOVA with Sidak's post hoc or Bonferroni post hoc test was performed for statistical analysis in GraphPad Prism version 8.0 software (GraphPad software). Differences which were below 0.05, considered significant and depicted with an asterisk (*) on each graph. Reported n is the number of animals/samples analyzed.

4 RESULTS AND DISCUSSION

Previous studies in the lab have identified a circadian clock in the mouse cochlea, associated with greater vulnerability to noise at nighttime (Meltser et al., 2014). This difference in noise sensitivity was related to neurotrophins, which signal the IHC synapse to noise damage. However, there is still very little knowledge about what maintains the synaptic integrity. Here, we examine the role of GCs in the circadian vulnerability to noise and find that they are involved in the auditory recovery from night noise trauma, but unlike neurotrophins, independently from synaptic recovery (Paper I). Therefore, we evaluated whether mice lacking glutamate transporter GLAST could be more suitable model for investigating the synaptic integrity influenced by circadian rhythms, since they have impaired glutamate uptake after noise exposure (Hakuba et al., 2000). We observed that indeed GLAST KO mice display characteristics of auditory synaptopathy in the absence of noise exposure (Paper II). Since glutamate transporters and circadian mechanisms appear to be involved in ototoxicity, we investigated whether the two combined could regulate the vulnerability to cisplatin, a major anti-cancer drug causing hearing loss. We found that GLAST KO mice are more susceptible to cisplatin when administered at nighttime, with a greater loss of synaptic ribbons and synaptic coupling (Paper III). We finally performed a systematic review and meta-analysis to identify human relevant genes related to cisplatin-mediated ototoxicity, where 8 polymorphisms of 5 genes were found to be significantly associated (Paper IV). Four of these five genes display circadian expression patterns in the cochlea suggesting they could be potentially involved in the circadian vulnerability to cisplatin ototoxicity in humans.

4.1 PAPER I: Glucocorticoid removal protects mouse cochlea from night noise exposure

In this study, we hypothesized that circadian vulnerability to noise trauma might be influenced by GC function, which are clock-controlled and have been shown to modulate auditory sensitivity to noise trauma (Canlon et al., 2007). Thus, we investigated the relationship between GC rhythms and the cochlear core clock genes in the response to noise trauma. First, we confirmed that SCN is responsible for the sustained rhythmicity in the cochlea. Next, we found that in the absence of BMAL1 protein there was a decline of *ex vivo* cochlear rhythmicity, which means that cochlear clock requires BMAL1 involvement for conserving a self-sustained clock machinery. In order to evaluate the role of GCs, which are established downstream mediators of SCN, we performed adrenalectomy (ADX) in mice and we exposed them to day or nighttime noise, where we observed that auditory function, 2 weeks after night noise

exposure, was almost fully restored in ADX mice but not in sham mice (**Figure 3A**). However, ADX did not rescue the presynaptic and postsynaptic structures after night noise trauma (**Figure 3B**).

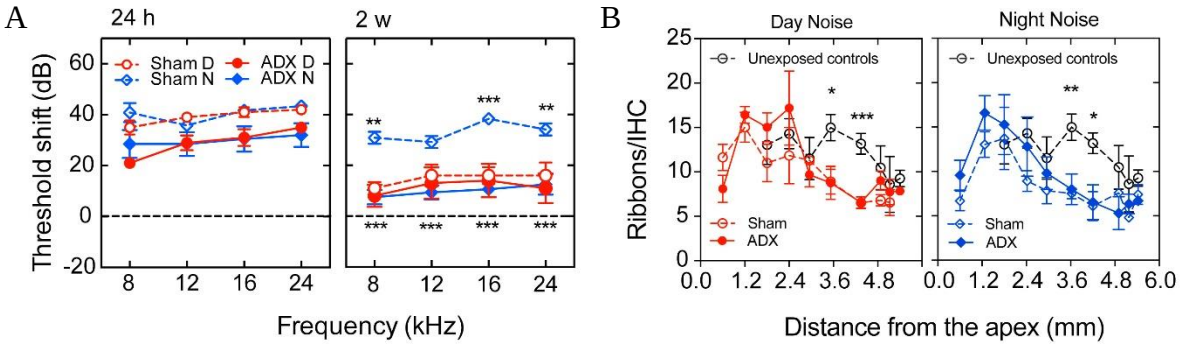

Figure 3: **Endogenous GCs control the vulnerability to night noise exposure without affecting the presynaptic ribbons.** (A) ABR threshold shifts from sham operated mice (open symbols) and ADX treated mice (filled symbols) at 24h and 2 weeks after day (09.00 am_day color) or night (09.00 pm_blue color) noise trauma. Results are mean values ± SEM, n = 5–8 (C). *p < 0.05, ** p < 0.01, ***p < 0.001; two-way ANOVA with Bonferroni post hoc analysis. (B) Quantification of synaptic ribbons along cochlear frequencies from apex to the base from unexposed control mice (black open circles), sham and ADX operated after day noise exposure (red circles) and after night noise exposure (blue diamonds), 2 weeks after the trauma. Results are mean values ± SEM, n = 1–7. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Sidak post-hoc analysis.

These findings pinpoint that GC ablation regulates the cochlear sensitivity to noise trauma at nighttime without influencing the presynaptic ribbons. Further, RNA sequencing analysis on the whole cochlea revealed genes, which are involved in neurotransmitter signaling and whose induction by noise was significant at nighttime, being completely abolished in ADX operated mice. In contrast, ADX did not impede gene induction after day noise exposure. In order to deepen in the molecular pathways that regulate the circadian vulnerability to noise in the absence of GCs, we identified circadian genes, which lost their rhythmicity after ADX, suggesting their involvement in the immune system and likely the regulation of inflammatory reactions within the cochlea. Last, we investigated the role of DEX, a synthetic glucocorticoid agonist, on cochlear rhythms and noise trauma. We observed that DEX has a differential outcome on cochlear rhythms depending on the time of the day that is administered. In order to evaluate which is the effect of DEX *in vivo*, we treated mice with DEX prior to day or night noise exposure and we found that DEX treatment protected mice exposed to day trauma but not to night noise trauma.

The findings of this study confirm that cochlear circadian rhythms are regulated by *Bmal1* and present a hierarchical connection between SCN, adrenal glands and the cochlear clock, which has never been identified before. The main outcome of this paper is that ADX protects mice from night noise damage but without saving the afferent neuron synapse, suggesting that GCs mediate the differential sensitivity to day or night noise exposure independently from ribbon

counts. Overall, the above findings indicate that the differential response to noise, depending on the time of the day, may implicate additional mechanisms, which have not been identified yet, such as SGN function or SV regulation. Another interesting observation of this study is the role of GCs in the involvement of pro-inflammatory signals including the chemokine *Ccl2*, involved in the trafficking of monocytes. Monocytes have been previously identified in the postnatal mouse cochlea (Hirose et al., 2005) but also accumulation of CX3CR1 macrophages after acoustic trauma (Sato et al., 2008). Our data suggests that mice are more sensitive to night noise because GCs predispose the cochlea to inflammatory reactions.

The most commonly used agent to treat hearing disorders is DEX (Wei et al., 2013). However, in this study we show that the time of the day when the drug is administered plays an essential role in determining its efficacy.

4.2 PAPER II: GLAST deficient mice are synaptopathic in the absence of noise trauma and show sound-evoked hyperactivity in the IC

Since in the first study we did not find a direct connection between GCs and synaptic damage, we sought to exacerbate the vulnerability of auditory synapse using mice deficient for GLAST and test its contribution into the circadian regulation of auditory function. Here, in this study, we investigated the influence of GLAST loss of function on sound induced brain activity. We first examined the hearing characteristics and the cochlear morphology of GLAST KO mice in the lack of noise exposure. We observed the pre-existence of cochlear synaptopathy since ABR wave 1 amplitude was significantly lower and the number of postsynaptic GluR2 receptors was reduced in the GLAST KO mice compared to their WT littermates. We further exposed GLAST WT and KO mice to a 24h 90 dB narrow-band noise. After noise exposure, paired synapses were almost abolished in the GLAST KO mice showing a clear exacerbation of glutamate excitotoxicity. We also assessed sound-evoked responses using MeMRI. The quantification presented a greater Mn^{2+} uptake in the IC of GLAST KO mice when compared to sham exposed mice. Such enhancement was not observed in WT mice (**Figure 4**).

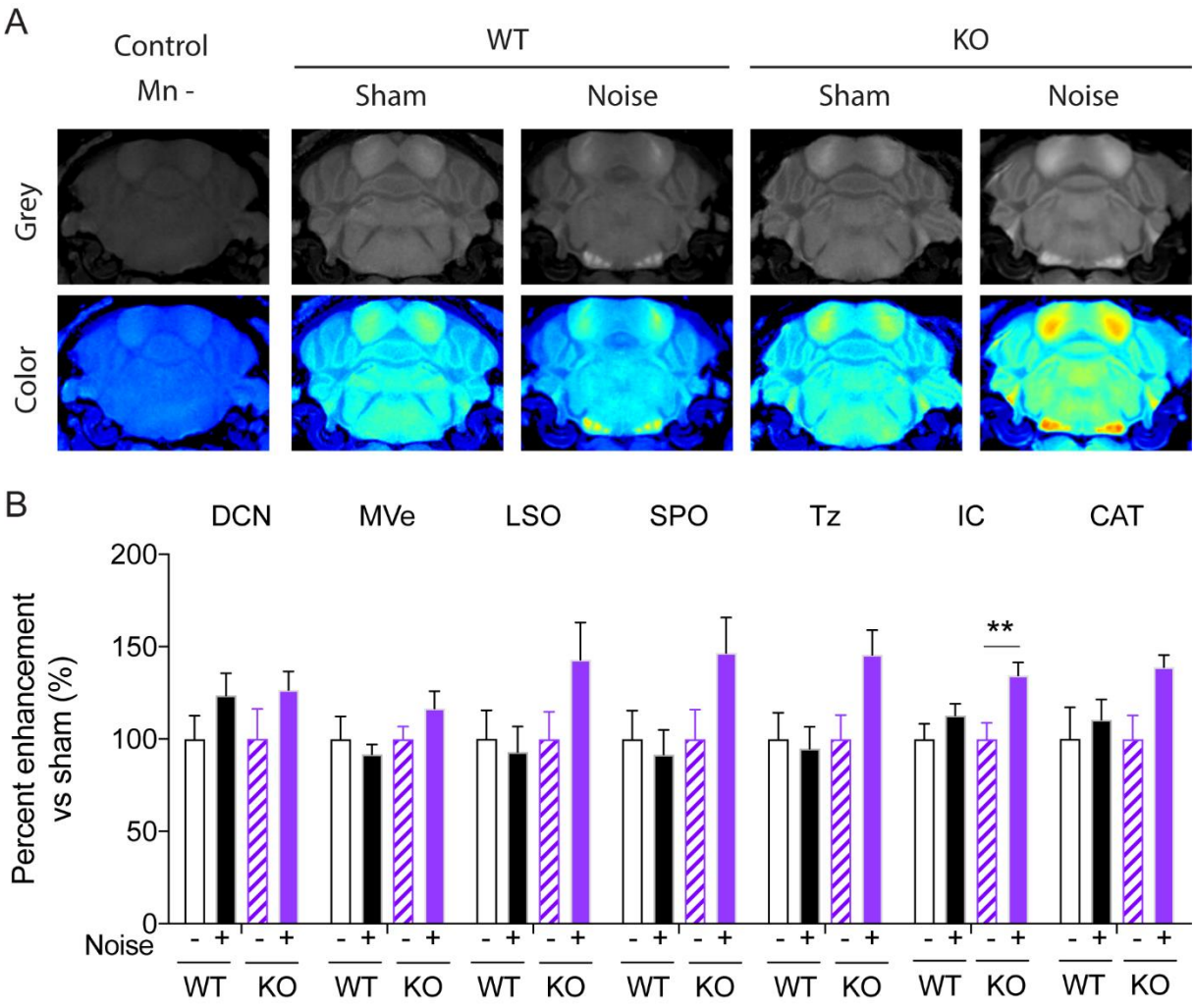

Figure 4: Sound evoked hyperactivity in different brain auditory regions of GLAST KO mouse identified with MeMRI and quantification of signal enhancement. (A) Representative MeMRI scans of GLAST WT and KO mice, sham or noise exposed (90 dB, 24h). Mn^{2+} uptake was measured in coronal sections. Warmer colors presented higher activity in the specific regions. (B) Quantification of the percent enhancement of the brain activity in the ascending pathway of auditory system (DCN, MVe, LSO, SPO, Tz, IC and CAT) in the GLAST KO compared to the WT mice. All signals normalized to CPu activity. Results are mean values ± SEM, n = 9-15, $^{**}p < 0.01$, two-way ANOVA with Sidak's post-hoc analysis.

Overall, our study displays evidence of a pre-existing cochlear synaptopathy in GLAST KO mice in the absence of noise exposure. This is associated with decreased ABR wave 1 amplitude and reduction of paired synapses in the absence of GLAST, despite unaffected ABR and DPOAE thresholds. It has been shown that the amount of glutamate levels in the perilymph of GLAST KO mice is double, compared to the one in the WT mice, proposing that such differences can cause changes in postsynaptic receptors even at baseline levels (Hakuba et al., 2000). The increased synaptic damage after sound overexposure was related centrally with an increased hyperactivity in the IC of GLAST KO mice. Notably, DCN did not present increased activity in GLAST KO mice as previously reported in CD-1 mice, although we used very

similar protocols (Yu et al., 2005). It remains unclear which is the specific contribution of GLAST function in the peripheral or central auditory structures.

4.3 PAPER III: Night cisplatin administration induces hearing loss accompanied by reduction of ribbons and synapses in GLAST KO mice

In order to investigate the circadian impact on cisplatin ototoxicity in GLAST KO mice, we treated them with specific dose of cisplatin (4 mg/kg) for 4 consecutive days, either at daytime (09.00 am) or nighttime (09.00 pm). We observed that night cisplatin administration caused hearing loss in GLAST KO mice without influencing the OHC function (**Figure 5**). Wave 1 amplitude was not affected more from cisplatin treatment since it was already reduced almost 50% in GLAST KO mice compared to the WT in baseline level, replicating what was shown in paper II. Moreover, night cisplatin administration caused greater loss of presynaptic ribbons and almost complete synaptic uncoupling in GLAST KO mice (**Figure 6**).

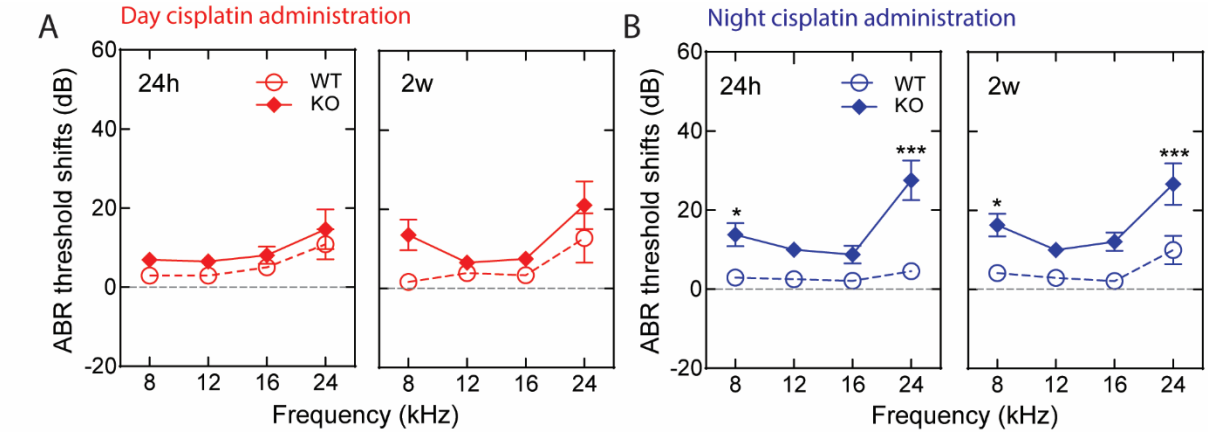

Figure 5: **Night cisplatin administration upregulates ABR threshold shifts of GLAST KO mice.** (A) ABR threshold shifts of GLAST WT (open circles) and GLAST KO mice (filled diamonds), 24h and 2 weeks after day cisplatin treatment. (B) ABR threshold shifts of GLAST WT and KO mice, 24h and 2 weeks after night cisplatin treatment. Results are mean values ± SEM; n=10-13. *p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA with Sidak's post hoc analysis.

The greater impact of night cisplatin treatment on hearing thresholds and synaptic damage was not due to higher bioavailability of the drug at night compared to day treatment, which was confirmed by performing ICP-MS in the different cochlear compounds and in the blood after day or night cisplatin administration. Interestingly, measurements in the blood showed greater abundance of platinum compounds after day administration, probably because of reduced detoxification in the inactive phase of the mouse. Platinum compounds in the OC/SGN and SV

remained the same either after day or night cisplatin treatment. These findings suggest the implication of other mechanisms in the higher influence of cisplatin at nighttime on hearing function in GLAST KO mice. We were further interested in evaluating whether cisplatin affects cochlear clock rhythms. We found that night cisplatin treatment changed the amplitude, phase and period of PER2 oscillations, something that did not occur after day cisplatin treatment. These findings show that cisplatin impacts on cochlear clock function at nighttime.

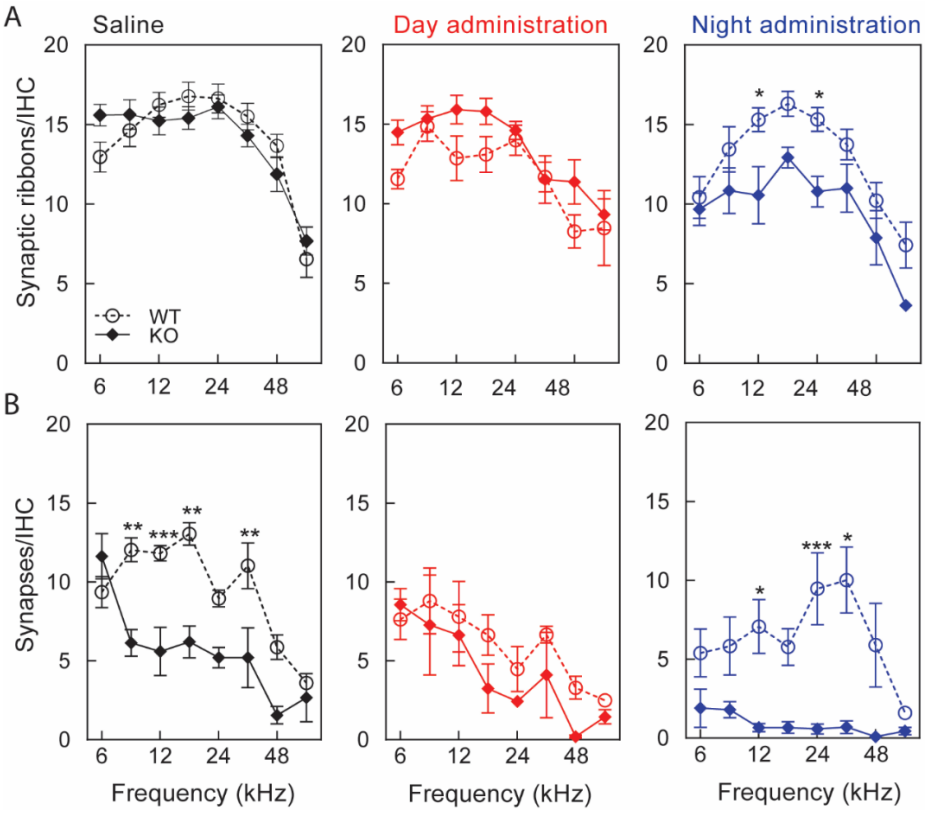

Figure 6: Night cisplatin administration reduces synaptic ribbons and completely abolishes synaptic pairing in GLAST KO mice. (A) Quantification from confocal images of synaptic ribbons in GLAST KO (filled diamonds) and WT (open circles) mice, 2 weeks after day (red) or night (blue) cisplatin treatment. Quantification of ribbons in saline treated GLAST KO (diamonds) and WT (circles) mice presented in black. (B) Calculation of the number of synaptic pairing in saline treated mice (showed in black), in day cisplatin treated group (red) and night cisplatin treated group (blue). Results are mean values ± SEM; n=1-13. *p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA with Sidak's post hoc analysis.

A previously published dataset from our own lab (used in Paper I as well) presented the expression of 5 mouse genes that are related to human cisplatin ototoxicity (paper IV). The four of these, *Acyp2*, *Lrp2*, *Sod2* and *Tpmt2*, which have an antioxidant role, or they are involved in metabolism, were found circadian with their peak expression occurring around night onset.

The findings of this study demonstrate the contribution of glutamate function in the circadian sensitivity to cisplatin-induced ototoxicity in mice and especially in the afferent synapse, which appears more vulnerable to night cisplatin treatment in mice, in the absence of GLAST.

Ototoxicity is a major side effect of cisplatin treatment in cancer patients (Rybak et al., 2007). For that reason, the contribution of chronopharmacology in cancer treatment could be important and may prove beneficial. We observe that mice with normal glutamate activity are not as vulnerable to circadian influences as the mice with glutamate deficiencies. Moreover, the obvious decline in paired synapses of GLAST KO mice after night cisplatin treatment was not related to similar changes in wave 1 amplitude, suggesting that there is not always a direct correlation between synaptic function and wave 1 amplitude as previously described (Fernandez et al., 2020; Kujawa et al., 2009). Our findings contrast those reported in rats, where cisplatin caused greater damage when administered at daytime (Bielefeld et al., 2018). In contrast, they come in agreement with previous studies in mouse brain extracts, where the expression of DNA repair protein XPA (xeroderma pigmentosum A) is higher during daytime, thus the repair of cisplatin-induced DNA damage is greater during the day (Kang et al., 2009). Probably, there are different circadian responses to ototoxic drugs depending on the species to which they are administered.

GLAST protein is not only expressed in IHC/synapse but also in other cochlear structures such as the satellite cells of SGNs and in the SV, which leads to the conclusion that additional mechanisms may be involved in the circadian vulnerability to cisplatin ototoxicity. While this study does not directly address the mechanism(s) underlying circadian cisplatin ototoxicity; future studies, leveraging on the models used in Paper I (SCN ablation and ADX), would help clarifying the contribution of clock components in the circadian vulnerability to cisplatin ototoxicity.

4.4 PAPER IV: Meta-analysis identified genetic polymorphisms associated with human cisplatin ototoxicity.

In this study we performed a systematic review followed by a meta-analysis of 30 previously published papers where we identified 8 polymorphisms in 5 genes associating with cisplatin ototoxicity (**Table 1**). The interesting observation from this systematic review is that individual studies with non-significant findings, when combined, reached enough power, showing an increased risk of developing cisplatin ototoxicity. This is the case for *LRP2* rs4668123, which highlights the need of using larger sample sizes for more robust results.

Table 1: Summary of all human genes and SNPs related to high risk of cisplatin ototoxicity. Genes that are followed by an asterisk have been identified as being circadian from mouse cochlea RNAseq data from study I.

Gene	SNP	OR	CI Low	CI High	Sample size	p (X2)	p of Fisher
ACYP2*	rs1872328	4.618	3.04	7.02	696	<0.0001	<0.0001
LRP2*	rs4668123	3.53	1.48	8.45	118	0.0026	0.0053
TPMT*	rs12201199	2.822	2.06	3.86	786	<0.0001	<0.0001
LRP2*	rs2075252	2.80	1.25	6.28	118	0.010	0.013
TPMT*	rs1142345	2.618	1.93	3.56	786	<0.0001	<0.0001
TPMT*	rs1800460	2.472	1.82	3.35	786	<0.0001	<0.0001
SOD2*	rs4880	1.917	1.01	3.61	177	0.04	0.05
COMT	rs9332377	1.553	1.18	2.05	847	<0.0001	<0.0001

The genes that we identified from repeated studies are mainly involved in antioxidant, metabolic or neurological pathways. More specifically, *ACYP2* plays a major role in Ca^{2+} homeostasis, which has been shown to participate in hair cell development (Xu et al., 2015). The *ACYP2* polymorphism showed the greatest risk for cisplatin ototoxicity, which should be considered in clinical cancer treatments. *LRP2* polymorphisms are also associated with high risk of cisplatin ototoxicity. *LRP2* or megalin is involved in sensorineural deafness and developmental retardation (Khalifa et al., 2015). TPMT, a methyltransferase, has an enzymatic activity which varies depending on the polymorphisms of *TPMT* gene. In this study, three polymorphisms of *TPMT* seem to affiliate with high risk of cisplatin induced ototoxicity. It was recently shown that HEI-OC1 cells derived from cochlea are more vulnerable to cisplatin treatment in the presence of a variant of *Tpmt* gene (Bhavsar et al., 2017).

Furthermore, one polymorphism of *COMT* has been identified as a significant risk factor. Mutations in *COMT* are involved in sensorineural hearing loss. Generally, there are strong indications that catecholamines potentially play a role in auditory function. The last important gene involved in cisplatin-mediated ototoxicity, is the antioxidant *SOD2,* which regulates ROS production and modifies mitochondrial function (Brown et al., 2015).

A genetic predisposition to cisplatin ototoxicity has been clarified and may help finding cancer patients, children or adults, with greater ototoxic risk. This could be a significant improvement to the discovery and development of otoprotective therapies in combination with cisplatin interventions. It is important to maintain cisplatin efficacy in cancer therapies. Thus, knowing

details about genetic predisposition could facilitate the advance of specific pathways being involved in protection against cisplatin ototoxicity.

5 CONCLUDING REMARKS

In this thesis, I evaluated the factors by which circadian rhythms modulate hearing impairment, focusing on two different models of auditory insults: noise exposure and cisplatin ototoxicity. This research demonstrated that GC function can modify NIHL at nighttime (the active phase of the mouse), possibly through mechanisms that involve inflammatory responses in the cochlea. Synaptic uncoupling by noise was not influenced by GCs. Moreover, these results highlight the interactions between endogenous and exogenous GCs in response to noise trauma. DEX, a synthetic GR agonist, was only effective when administered at daytime, when endogenous GC levels are low. Thus, these findings suggest that the appropriate timing of drug administration (chronopharmacology) is important for hearing protection.

The contribution of the glutamate transporter GLAST in auditory function and synaptic integrity was further evidenced. Loss of GLAST function led to a reduction of synaptic coupling in the absence of noise or cisplatin. Additionally, mice deficient in GLAST, presented elevated sound-evoked neural activity in the IC. These findings pinpoint to the fact that GLAST plays an essential role in auditory function, and triggers compensatory gain mechanisms in the central auditory pathway.

Moreover, GLAST depletion altered the vulnerability to cisplatin induced ototoxicity depending on the time-of-the-day when administered. After night treatment, GLAST deficient mice had disrupted pre- and post-synaptic coupling.

Last, a systematic review and meta-analysis of genetic studies investigating susceptibility to cisplatin ototoxicity revealed several polymorphisms associated with cisplatin ototoxicity in humans. Four of these genes were identified as being circadian when using the RNAseq data from mouse cochlea, suggesting their potential involvement in the circadian vulnerability to cisplatin.

Overall, the results of this thesis highlight different aspects of circadian regulation in the auditory system and suggest that a chronopharmacological approach in therapeutic strategies may prevent hearing loss following either noise trauma or cisplatin treatment.

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